Organic Acid Production by Basidiomycetes
I. Screening of Acid-Producing Strains

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ABSTRACT

Takao, Shoichi (Hokkaido University, Sapporo, Japan). Organic acid production by Basidiomycetes. I. Screening of acid-producing strains. Appl. Microbiol. 13:732-737. 1965.—Sixty-seven strains belonging to 47 species of Basidiomycetes were examined for their acid-producing abilities in glucose media, in both the presence and absence of CaCO$_3$, in stationary and shake cultures. Some strains were found to produce large quantities of oxalic acid. The oxalic acid-producing strains could be separated into two groups. Strains of one group (mostly brown-rot fungi) were able to produce oxalic acid, regardless of whether CaCO$_3$ was present in the medium. Strains of the other group (mostly white-rot fungi) were characterized by their ability to produce oxalic acid only when CaCO$_3$ was added to the medium. With the latter group, shake-culturing was generally more effective than stationary culturing in respect to acid production. In the CaCO$_3$-containing media, Schizophyllum commune, Merulius tremellosus, and Porodiscus pendulus were found to produce substantial amounts of L-malic acid as a main metabolic product, along with small quantities of oxalic and other acids in shake cultures. Especially, S. commune and M. tremellosus may be employed as malic acid-producing species.

Basidiomycetes comprise a very large number of species within the Eumycetes. Extensive studies concerning the metabolic products of fungi and yeasts have been undertaken. On the other hand, many of the biochemical investigations of Basidiomycetes have been carried out with emphasis on the nutritional, medicinal, or toxic effects of materials present in their fruiting bodies. Moreover, there are relatively few reports on their metabolic products, with the exception of oxalic acid, antibiotics, and certain other compounds.

Based on these facts, many kinds of Basidiomycetes were cultured to compare their metabolic features, especially acid-producing abilities, and to detect valuable end products. As a result, it was found that some strains produced large amounts of oxalic acid, and some others had the capacity to produce substantial amounts of L-malic acid as a main product from glucose.

De Bary (1886) first found potassium oxalate deposited in Peziza sclerotinia, and later calcium oxalate, or free oxalic acid, was observed in several kinds of Basidiomycetes (Zellner, 1907). On the other hand, after Sakaguchi and Nakao (1931) obtained oxalate in the culture solution of Cortinellus shiitake (present name, Cortinellus edodes), many species of Basidiomycetes were studied for oxalic acid production, including Armillaria mellea (Hamada, 1940), Coniophora cerebella (Birkimshaw, Findlay, and Webb, 1940), Merulius niveus, M. tremellosus, M. confuens, Fomes annosus (Nord and Vitucci, 1947), Poria vaporaria (Fukinbara, 1948; Shimazono, 1951), Kamibayashi and Nakatai, 1960), Merulius laeryms, Marasmius chordalis (Smith, 1949), Polyopus aniceps (Perlman, 1949), Fomitopsis pinicola, Piptoporus betulinus (Shimazono, 1951), Kamibayashi and Nakatai, 1960), Fomitopsis sp., Phaeolus schweinitzii, Stereum fractulosum, Trametes albida, T. dickinsi, Tyromyces sambucicola (Shimazono, 1951), Trametes cinabarina (De Stevens, De Baun, and Nord, 1951), Corticium centrifugum (Nagata and Hayashi, 1956; Kamibayashi and Nakatai, 1960), Coniophora puteana, Daedalea quercina, Fomitopsis officinalis, Laetiporus sulphureus, Polyporus palustris (Kamibayashi and Nakatai, 1960), Pleurotus ostreatus (Tsao, 1963a), and Agaricus campestris (Tsao, 1963b).

However, the production of organic acids, other than oxalic acid, from sugars by Basidiomycetes has scarcely been reported. In this connection, there is an undetailed report by Tachibana and Shiodo (1962) on malic acid production by Schizophyllum commune.

In this paper, the screening of acid-producing...
strains of Basidiomycetes and the identification of organic acids are discussed.

Materials and Methods

Organisms. Sixty-seven strains belonging to 47 species of Basidiomycetes (Tables 1 and 2) were supplied through the generosity of the Fermentation Research Institute, Chiba, and the Hokkaido Forest Products Research Institute.

Cultural procedure. All of the strains were grown on potato-glucose-agar slants, containing 5% malt sprouts extract, at 27 C for 10 days. They were inoculated into a mixture of 3 g of oak sawdust, 2 g of rice bran, and 5 ml of tap water in 100-ml Erlenmeyer flasks. After 10 to 14 days of incubation, they were transferred to 40 ml of medium in 150-ml Erlenmeyer flasks, then stationary-cultured at 27 C for 21 days. Each strain was also cultured in 50 ml of medium in 500-ml shake flasks on a reciprocating shaker at 27 C for 7 days. The medium contained 5% glucose, 0.2% peptone, 0.5% KH₂PO₄, 0.25% MgSO₄·7H₂O, 0.2% yeast extract, and 1% malt sprout extract; 2% CaCO₃ was added to some of the preparations.

Analytical methods. The acidity in the culture solution, in the absence of CaCO₃, was determined by titration with 0.1 N NaOH. Since the insoluble calcium salt appearing in the CaCO₃-containing broth consisted almost entirely of calcium oxalate, it was treated with hot 20% sulfuric acid, and the acid liberated from calcium was estimated as oxalic acid by titration with standard KMnO₄ solution. Soluble calcium remaining in the filtrate was determined by precipitation of calcium as oxalate and subsequent titration with KMnO₄ solution. Glucose was measured by the method of Somogyi (1945). Mycelial weight was determined after drying overnight in an oven at 100 C.

Elution analysis of organic acid was made by the method of Bulen, Varner, and Burrell (1952). After the filtrate containing soluble calcium salt was treated with ion-exchange resins (Amberlite IR 120), the acid sample was chromatographed on a column of silica gel prepared from Mallinekrod's silicic acid (specially prepared for chromatographic analysis), with a series of n-butanol-chloroform solvents (100 ml of 5%, 135 ml of 15%, 100 ml of 25%, and 300 ml of 35% n-butanol-chloroform), and 3.3 ml of each effluent was titrated by addition of 0.005 N NaOH in the presence of phenol red indicator. Paper chromatography of acid was carried out by use of the following two solvent systems: phenol-formic acid-water (75:1:25; Block, Durrum, and Zweig, 1958) and n-butanol-formic acid-water (4:1.5:1; Kawano and Kawabata, 1953).

Results and Discussion

Acid production in the medium which did not contain CaCO₃. The results obtained in the media not containing CaCO₃ are shown in Tables 1 and 2. Titratable acidity is presented as the number of milliliters of 0.1 N NaOH required to neutralize 10 ml of culture solution. Most of the strains which produced high acidity in stationary cultures gave similar results in shake cultures. Among them, a few strains, such as Laetiporus sulphureus and Phellinus yucatanensis, showed much lower acidity in shake cultures than in stationary cultures. This may be related to the poor growth of these strains under shake-culture conditions.

Oxalic acid-producing strains. The culture solutions of strains which gave high acidity in the previous experiment were subjected to paper chromatography. Only one spot was recognized with every strain in both solvent systems, and each Rₚ value, 0.18 or 0.06, was identical with that of oxalic acid. Acid crystals, mp 189 to 190 C, were obtained by extracting the broth with ether. There was no depression in the melting point when the crystals were mixed with authentic oxalic acid (mp 190 C).

Acid liberated from insoluble calcium salts, which were produced in large amounts by some strains in the CaCO₃-containing medium, was

| Table 1. Acid-producing strains of Basidiomycetes in media without CaCO₃ |
|-----------------|-----------------|-----------------|
| Species         | Strain          | Stationary culture (21 days) | Shake culture (7 days) |
|                 |                 | 0.1 N NaOH/10 ml | Glucose consumed | Mycelial wt/100 ml | 0.1 N NaOH/10 ml | Glucose consumed | Mycelial wt/100 ml |
|                 |                 | ml | % | g | ml | % | g |
| Coniophora puteana | 9328 | 6.6 | 44 | 1.0 | 14.4 | 42 | 0.3 |
| Corticium rolfsii | 9404 | 5.8 | 98 | 2.2 | 5.6 | 85 | 3.0 |
| Fontopus officinalis | 9354 | 12.8 | 53 | 0.6 | 4.9 | 25 | 0.4 |
| Gloeophyllum trabeum | 9315 | 2.9 | 48 | 0.6 | 5.2 | 49 | 0.4 |
| Laetiporus sulphureus | 9304 | 9.2 | 20 | 0.3 | 2.4 | 37 | 0.2 |
| Phellinus yucatanensis | 9377 | 10.9 | 44 | 0.6 | 2.3 | 34 | 0.2 |
| Polyporus palustris | 9378 | 4.2 | 60 | 1.6 | 12.6 | 33 | 0.3 |
| Poria vaporaria | 9320 | 9.3 | 34 | 0.7 | 21.6 | 49 | 0.9 |
| P. vaporaria | 9381 | 6.5 | 78 | 1.5 | 19.0 | 38 | 0.9 |
also identified as oxalic acid by means of paper chromatography and melting point.

Oxalic acid yields by these strains under four types of cultural conditions are compared in Table 3. It was possible to classify the strains into two groups: strains in one group were able to produce oxalic acid, whether or not CaCO₃ was present in the medium, and strains belonging to the other group produced the acid only when CaCO₃ was added to the medium.

It is well known that there are two groups, “brown-rot fungi” and “white-rot fungi,” among wood-destroying Basidiomycetes. Shimazono (1951) reported that many species of brown-rot fungi accumulate free oxalic acid in culture media without CaCO₃, whereas most white-rot fungi can accumulate the acid only when the reaction of the medium becomes alkaline or CaCO₃ is added. Shimazono (1955) also indicated that most of the species of white-rot fungi had the ability to decompose oxalic acid, and that the decomposition of the acid was due to the presence in the mycelium of “oxalic acid decarboxylase” which specifically affected decarboxylation of oxalic acid at the acidic pH.

Most species of the first group in Table 3 are brown-rot fungi. On the other hand, most of the species in the second group, such as *Coriolus versicolor*, *Elfvingia applanata*, and others, are white-rot fungi (Henni and Akai, 1945; Shimazono, 1951). The results of this experiment are, therefore, in good agreement with those obtained by Shimazono. Furthermore, it was recognized that the accumulation of oxalic acid, even by strains which did not necessarily require CaCO₃ for acid production, was generally increased by the addition of CaCO₃, and that shake culture was more favorable than stationary culture for oxalic acid production by the strains belonging to the second group.

### Table 2. Strains of Basidiomycetes which seldom produced acid in media without CaCO₃

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Armillaria mellea</td>
<td>9323</td>
</tr>
<tr>
<td>Bjerkaandra adusta</td>
<td>9325</td>
</tr>
<tr>
<td>Collubia velutipes</td>
<td>9309</td>
</tr>
<tr>
<td>C. velutipes</td>
<td>9327</td>
</tr>
<tr>
<td>Coriolus consors</td>
<td>9316</td>
</tr>
<tr>
<td>C. consors.</td>
<td>9401</td>
</tr>
<tr>
<td>C. hirsutus</td>
<td>9332</td>
</tr>
<tr>
<td>C. polyzonus</td>
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<td>C. versicolor</td>
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<td>9337</td>
</tr>
<tr>
<td>C. versicolor...</td>
<td>9415</td>
</tr>
<tr>
<td>Corticium caeruleum</td>
<td>9388</td>
</tr>
<tr>
<td>C. sasakii</td>
<td>9406</td>
</tr>
<tr>
<td>C. sasakii</td>
<td>9407</td>
</tr>
<tr>
<td>Cortinellus edodes</td>
<td>9306</td>
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<tr>
<td>C. edodes</td>
<td>9307</td>
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<td>9341</td>
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<tr>
<td>C. edodes</td>
<td>9344</td>
</tr>
<tr>
<td>Cryptoderma yamanoi</td>
<td>9348</td>
</tr>
<tr>
<td>Daedaleopsis stryacina</td>
<td>9349</td>
</tr>
<tr>
<td>Elfvingia applanata</td>
<td>9350</td>
</tr>
<tr>
<td>Favolus arcarius</td>
<td>9351</td>
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<tr>
<td>Fomitopsis annosa</td>
<td>9353</td>
</tr>
<tr>
<td>Ganoderma lucidum</td>
<td>9357</td>
</tr>
<tr>
<td>Gloeophyllum sepiarium</td>
<td>9358</td>
</tr>
<tr>
<td>G. sepiarium</td>
<td>9397</td>
</tr>
<tr>
<td>Grifola frondosa</td>
<td>9322</td>
</tr>
<tr>
<td>G. frondosa</td>
<td>9360</td>
</tr>
</tbody>
</table>
conditions (Table 4). Any acid accumulation by these strains was not detected in media without CaCO₃.

The culture solutions containing soluble calcium salts were treated with cation-exchange resin, and were then subjected to column chromatography. The location of each acid peak on the chromatogram was compared with that obtained from the standard survey column. The chromatogram obtained with every strain of Schizophyllum commune was very similar; the result with strain 9384 is shown in Fig. 1. It is obvious that S. commune produces considerable amounts of malic acid in addition to relatively small quantities of fumaric and succinic acids. Pordisculus pendulus was also found to produce the same acids as S. commune (Fig. 2). Merulius tremellosus produced acetic acid in addition to fumaric, succinic, and malic acids (Fig. 3). Each peak effluent was collected and chromatographed on paper in two solvent systems. Their R_f values were identical with those of fumaric, succinic, and malic acids.

It appeared that all five of these strains produced malic acid as a main product. Therefore, the culture solutions were acidified and extracted with ether in a continuous extractor in the usual manner. After recrystallization of the extract from ethyl acetate by addition of petroleum ether, the acid crystals melted at 97 to 100 C (L-malic acid, mp 99 to 100 C).

Rotation of malic acid produced by S. commune 9384 and M. tremellosus 9371 was determined with a Hitachi polarimeter (Table 5).
FIG. 1. Chromatogram of acids produced by Schizophyllum commune 9384.

FIG. 2. Chromatogram of acids produced by Porodisculus pendulus 9379.

FIG. 3. Chromatogram of acids produced by Merulius tremellosus 9371.

TABLE 5. Rotation of malic acid produced by Schizophyllum commune and Merulius tremellosus

<table>
<thead>
<tr>
<th>Malic acid prepn</th>
<th>Final concn</th>
<th>Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. commune 9384</td>
<td>0.1544</td>
<td>$[\alpha]_D^0 = +1360^\circ$</td>
</tr>
<tr>
<td>M. tremellosus 9371</td>
<td>0.1416</td>
<td>$[\alpha]_D^0 = +1370^\circ$</td>
</tr>
<tr>
<td>L-Malic acid</td>
<td>0.25</td>
<td>$[\alpha]_D^7 = +1335^\circ$</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>$[\alpha]_D^7 = +1407^\circ$</td>
</tr>
</tbody>
</table>

* Krebs and Eggleston (1943).

five strains, based on available glucose, are indicated in Table 4. Each acid was estimated from titratable acidity for the peak effluent of chromatography. Table 4 suggests the possibility that S. commune and M. tremellosus may be employed as malic acid-producing species. Information relating to the production of higher yields of malic acid by these species of Basidiomycetes, by altering cultural conditions, will be published elsewhere. In this connection, the production of L-malic acid from sugar by Basidiomycetes has not been observed, with the exception of an undetailed report on S. commune (Tachibana and Shiode, 1962).

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LITERATURE CITED


